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MEASUREMENT OF ANTIFOLATE DRUGS BY MICROBIOLOGICAL ASSAY.(U)
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FINAL REPORT

MEASUREMENT OF ANTIFOLATE DRUGS BY MICROBIOLOGICAL ASSAY

Carl C. Smith, Ph.D.
Principal Investigator

Clara S. Genther, M.S.

July 1, 1975 to June 30, 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Washington, D. C. 20314

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Department of Environmental Health
University of Cincinnati College of Medicine
Cincinnati, Ohio 45267

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This report is a compendium of the two reports prepared under Contract DADA 17-73-C-3151 for the period July 1, 1975 to June 30, 1976. A brief summary of the most important findings is included in the abstract section of the DD1473.

Carl C. Smith

Carl C. Smith, Ph.D.
Principal Investigator

December 28, 1976

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The results of the work described include:

Report No. 13: A detailed description of the methods used to calculate the plasma levels of an antifolate compound from data obtained with the Streptococcus faecium assay is presented. Data, graphs and calculations are presented for 8 samples ranging in antifolate content from 43.8 to 0.005 µg/ml.

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Interim Report No. 13

Measurement of Antifolate Drugs by Microbiological Assay Methods Used to Calculate the Concentrations of Antifolate Compound in Plasma

Carl C. Smith, Ph.D.
Principal Investigator

Clara S. Genther, M.S.

February 10, 1976

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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Plasma effect, Plasma levels, Streptococcus faecium, microbiological assay, antifolate, calculations, methods

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A detailed description of the methods used to calculate the plasma levels of an antifolate compound from data obtained with the Streptococcus faecium assay is presented. Data, graphs and calculations are presented for 8 samples ranging in antifolate content from 43.8 to 0.005 µg/ml.

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Methods Used to Calculate the Concentrations of Antifolate Compounds in Plasma

The purpose of this report is to detail the methods by which the level of an antifolate compound in a plasma sample is calculated from the results of the antibacterial assay using *Streptococcus faecium*. In order to make clear how the data are obtained an outline of the assay procedure is included. Examples of a wide range of levels are presented.

The procedure used in the assay is divided into two parts. These are:

(1) A preliminary assay in which a rough determination of the level of antifolate is made. This is done by diluting the plasma 1/10, 1/100 and 1/1000 with water and adding 1 ml quantitites of each dilution to tubes of 10 ml final volume. Thus the quantities of plasma in each tube are 0.1, 0.01 and 0.001 ml respectively. The examples in Table 1 show a wide range of typical results obtained in such a preliminary assay.

(2) The principal assay is performed with twofold dilutions of plasma over a range determined from the preliminary assay. These dilutions are chosen to allow a range of growth of the test organism from Klett readings of 0-300 where possible. Where the level of antifolate is too low to cause much inhibition of growth, e.g. samples E,F,G and H, the plasma is added to the tubes in quantities ranging up to 1.0 ml of undiluted plasma. The dilutions used for the samples given in Table 1 and the growth results of the principal assay are presented in Table 2.

Standard plasma samples in which known amounts of the antifolate are added to plasma and diluted in the same manner as the unknown samples are included in each principal assay. The range of concentrations of these standards is determined by inspection of the preliminary results. Since we are showing a wide range of samples here the results for a wide range of standards are given in Table 3.

When all of the assay data have been obtained graphs are prepared of the standard readings and of the unknown sample readings. Semi-logarithmic paper is used putting the ml of plasma per tube* on the logarithmic scale and the Klett reading of growth on the arithmetic scale. The results with the standards have been plotted in Figure 1. When the concentration of antifolate is large enough the effects of plasma on growth and drug action are diluted out (see 10, 1 and 0.5 µg/ml), producing parallel curves. Comparison of these parallel curves is made at the point of 50% of the growth of the test organism in medium containing no drug or plasma. This IC50 occurs at a Klett reading of 150. If a horizontal line is drawn at the 50% growth point (Klett 150) the curves for these larger concentrations of drug will cross it at points proportional to the drug concentration. Thus in this set of data the point for the 10 µg/ml standard is 0.00057 ml plasma; for 1 µg/ml 0.0058 and for 0.5 µg/ml 0.012. With lower concentrations of drug and consequently smaller dilutions of the plasma there are effects of plasma upon the readings.

*We routinely plot ml plasma per tube since it is the same as the dilution made on the plasma and doesn't involve further calculation. If one wishes to use plasma/ml medium the numbers would be 0.1 of these and the same relationships hold throughout.

Thus instead of the 0.058 which one would expect from the 1 µg/ml data, the graph of the dilutions of the 0.1 µg/ml standard crosses the Klett 150 line at 0.074 ml plasma. Therefore, concentrations of antifolate near 0.1 µg/ml should be calculated from the 0.1 µg/ml standard. As the concentrations of antifolate in the plasma drop below the 0.1 µg/ml level larger amounts of plasma must be added to the tubes and there is an increasing effect of plasma. This is because at the higher concentrations, plasma has both a growth inhibitory and a drug antagonizing effect. For the detailed description of these effects see Interim Report No. 1. Therefore, at concentrations of antifolate at 0.1 µg/ml and below an unknown sample must be compared with a standard close to its own concentrations.

The results obtained with the unknown samples are plotted in the same way as the standards. These are shown in Figure 2. It can be seen that Samples A, B and C fall into the high concentrations of antifolate group which when assayed are diluted beyond the point where plasma itself exerts any effect. Therefore, for these samples the concentration of drug in the plasma is obtained by comparing the point where the curve of the unknown crosses the Klett 150 line (IC50) and the point where the curve of the nearest standard crosses the same line using the following equation:

$$\frac{\text{Concentration of standard}}{\text{Concentration of unknown}} = \frac{\text{IC50 unknown}}{\text{IC50 standard}}$$

The proportion is an inverse one since the larger the number of ml of plasma required for the IC50 the smaller the concentration of drug will be.

For Sample A the Klett 150 point (IC50) is 0.00013 ml plasma. This is calculated against the nearest standard (10 µg/ml - 0.00057) in the following manner.

$$\frac{10}{X} = \frac{0.00013}{0.00057}$$

$$X = \frac{10 \times 0.00057}{0.00013} = \frac{0.0057}{0.00013} = 43.8 \text{ µg/ml}$$

Thus Sample A exerts a growth inhibiting effect equal to 43.8 µg/ml antifolate.

For Sample B the IC50 point is at 0.00094. The calculations are similar to A.

$$\frac{10}{X} = \frac{0.00094}{0.00057}$$

$$X = \frac{0.0057}{0.00094} = 6.1 \text{ µg/ml}$$

Thus the value for sample B is 6.1 µg/ml antifolate.

For Sample C the IC50 is 0.0037. This curve is nearer the 1 µg/ml standard which crosses at 0.0058. The calculations are:

$$\frac{1}{X} = \frac{0.0037}{0.0058}$$

$$X = \frac{0.0058}{0.0037} = 1.6$$

Thus sample C has 1.6 µg/ml antifolate.

Sample D whose IC50 is 0.04 ml plasma is nearest the 0.1 µg/ml control standard which crosses at 0.074. The calculations are:

$$\frac{0.1}{X} = \frac{0.04}{0.074}$$

$$X = \frac{0.0074}{0.04} = 0.19 \text{ µg/ml}$$

Thus sample D has 0.19 µg/ml antifolate.

Sample D is the last one which can be compared directly with a standard at Klett 150. The estimation of the level of antifolate in the remaining samples is made by comparing the position of the graph of the sample with that of the nearest standard. The lines of the standards have been dotted into Figure 2 to show the relationships. Sample E is estimated to have 0.08 µg/ml; Sample F, 0.06 µg/ml; Sample G, 0.03 µg/ml and H, 0.005 µg/ml. These calculations are summarized in Table 4.

One other aspect of this method deserves documentation and that is the reproducibility of the assay data. Our studies on plasma levels of WR-158,122 and WR-99,210 provided the opportunity to evaluate replicate assays both with and without plasma. Similarly over a 5-year period we have over 100 assays of pyrimethamine. These data have been listed in Table 5 which includes mean, S.D., S.E., and range of our replicate assays.

SUMMARY

A detailed description of the methods used to calculate the plasma levels of an antifolate compound from data obtained with the *Streptococcus faecium* assay is presented. Data, graphs and calculations are presented for 8 samples ranging in antifolate content from 43.8 to 0.005 µg/ml.

Table 1

Results of a Typical Preliminary Assay

ml plasma per tube	Sample: Klett reading of growth							
	A	B	C	D	E	F	G	H
0.1	0	0	5	50	150	190	250	290
0.01	0	2	60	270	300	300	300	300
0.001	0	140	260	300	300	300	300	300

Table 2

Results of a Typical Principal Assay

ml plasma per tube	Sample A	B	C	D	E	F	G	H
	Klett reading of growth							
1.0					102	135	161	215
0.5				0	105	135	170	253
0.25				10	115	140	215	274
0.1				50	150	195	250	290
0.05			0	125	200	250	290	
0.025			10	200	255	290		
0.01		0	55	275	290			
0.05		0	120	300				
0.0025		5	190					
0.001	0	143	265					
0.0005	0	220	295					
0.00025	8	275						
0.0001	208	295						
0.00005	275							
0.000025	295							
0.00001	300							

Table 3

Assay Data on Plasma Standards

µg antifolate added to tube	10 µg/ml* ml plasma per tube	Klett	1 µg/ml ml plasma per tube	Klett	0.5 µg/ml ml plasma per tube	Klett	0.1 µg/ml ml plasma per tube	Klett
10	1.0	0	-	-	-	-	-	-
1	0.1	0	-	-	-	-	-	-
0.1	0.01	0	0.1	0	-	-	1.0	0
0.05	0.005	0	0.05	0	0.1	0	0.5	9
0.025	0.0025	34	0.025	35	0.05	42	0.25	25
0.01	0.001	23	0.01	15	0.02	31	0.1	115
0.005	0.0005	180	0.005	187	0.01	193	0.05	191
0.0025	0.00025	255	0.0025	258	0.005	248	0.025	250
0.001	0.0001	290	0.001	295	0.002	285	0.01	278
	0.07 µg/ml		0.05 µg/ml		0.025 µg/ml		0.01 µg/ml	
0.07	1.0	104	-	-	-	-	-	-
0.05	0.5	110	1.0	140	-	-	-	-
0.025	0.25	127	0.5	160	1.0	178	-	-
0.01	0.1	185	0.2	180	0.4	185	1.0	195
0.005	0.05	225	0.1	225	0.2	220	0.5	215
0.0025	0.025	260	0.05	260	0.1	250	0.25	260
0.001	0.01	290	0.02	295	0.04	290	0.1	280
	0.0 µg/ml							
0.0	1.0	230						
0.0	0.5	260						
0.0	0.25	285						
0.0	0.1	300						
0.0	0.0	305						

*µg of antifolate per ml in standard plasma.

Table 4

Summary of Calculations of Concentrations of Antifolate in 8 Samples of Plasma

Sample	Standard		Unknown	
	Conc. µg/ml	IC50*	IC50	Conc. µg/ml
A	10	0.00057	0.00013	43.8
B	10	0.00057	0.00094	6.1
C	1	0.0058	0.0037	1.6
D	0.1	0.074	0.04	0.19
E	0.07	-	-	0.08
F	0.05	-	-	0.06
G	0.025	-	-	0.03
H	0.01	-	-	0.005

*IC50 - ml plasma exerting a 50% growth inhibitory effect on test organism.

Table 5

Reproducibility of Replicate Drug Analyses
In the Presence or Absence of Plasma

Concentration	No of Dtns.	IC50	S.D.	S.E.	Range
			WR-158,122		
1 µg/ml (drug only)	12	0.0032	0.00028	0.00008	0.0028 - 0.0037
1 µg/ml plasma	8	0.0027	0.00032	0.00012	0.0023 - 0.0030
0.1 µg/ml plasma	8	0.032	0.0052	0.0019	0.025 - 0.042
			WR-99,210		
1 µg/ml (drug only)	6	0.0019	0.00039	0.00016	0.0016 - 0.0025
1 µg/ml plasma	6	0.0018	0.00046	0.00019	0.0014 - 0.0025
0.1 µg/ml plasma	5	0.0175	0.0025	0.0011	0.0145 - 0.021
			Pyrimethamine		
1 µg/ml	>100	0.022	0.002	0.0002	0.015 - 0.028

Figure 1

Assay Data on Plasma Standards

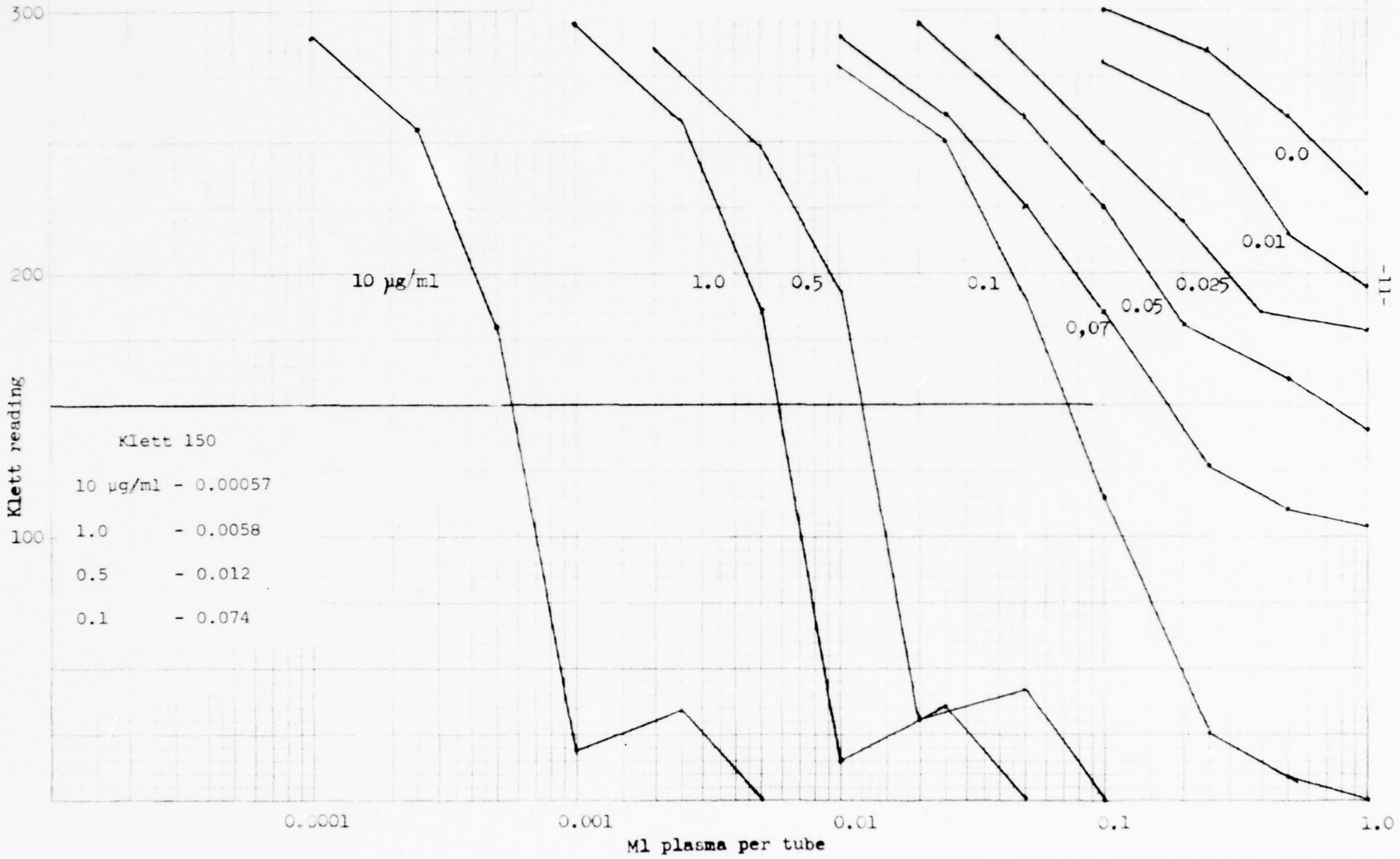
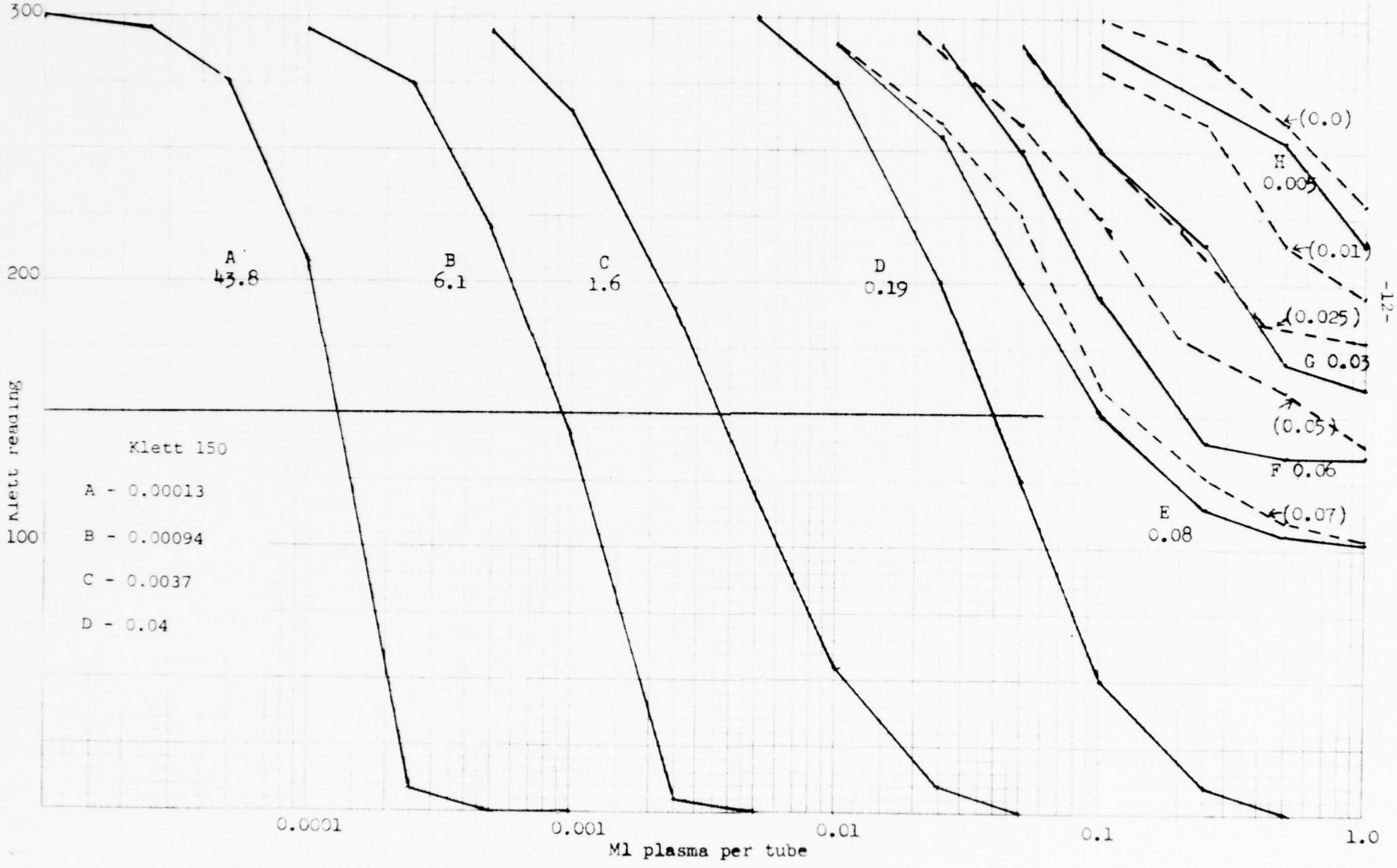


Figure 2

Data for Samples and Standards (--- from Fig.1)



Appendix A

Microbiological Assay for 1-(2,4,5-trichlorophenoxypropyloxy)-1,2-dihydro-2,2-dimethyl-s-triazine (WR-99,210) in Plasma

The microbiological assay for 1-(2,4,5-trichlorophenoxypropyloxy)-1,2-dihydro-2,2-dimethyl-s-triazine (WR-99,210) is carried out as follows. The test organism, Streptococcus faecium (ATCC 8043), is maintained on yeast extract agar and transferred twice in Flynn medium. The second transfer is washed and suspended in sterile saline to form a turbidity of 50 in the Klett photoelectric colorimeter. 0.1 ml of this suspension is used as inoculum for each tube.

The medium used for testing is that of Flynn et al., (1) except that folic acid and glucose are sterilized separately and added aseptically at the time of the test. The medium is tubed in 8 ml quantities and sterilized. One ml containing 0.02 µg folic acid in 25% glucose solution is added to each tube at the time of testing. Stock drug solutions if not prepared in a self-sterilizing solvent are sterilized by membrane filtration. If plasma samples have any considerable amounts of turbidity sterility checks are made by subinoculating into nutrient broth, by gram stains and by staining for oil droplets. The addition of one ml quantities of drug or plasma dilutions in water brings the total volume to 10 ml. The tubes are incubated at 37°C for 22 hours and the growth is measured as turbidity in the Klett photoelectric colorimeter.

In order to provide relative concentrations of drug and plasma (serum) encompassing the range that is encountered in a typical assay, solutions of the drug containing 0.001, 0.01, 0.1 and 1.0 µg/ml of the drug in plasma are prepared. These matrix standards are diluted for assay in the same manner as used for the unknown plasma (serum) samples. A preliminary assay is run using tenfold dilutions of the test samples to establish the range of activity of the drug present. A quantitative assay with twofold dilutions is then run in the range determined by the preliminary assay. The calculations of the concentration of drug in the plasma samples is made by comparison of the growth effects with those of standards containing the same amounts of plasma.

These methods and the range of results obtained are detailed in Interim Report No. 1 (2).

1. Flynn, L.M., Williams, V.B., O'Dell, B.L. and Hogan, A.G., Anal. Chem. 23:180-185, 1951.

2. Smith, C.C. and Genther, C.S., Interim Report No. 1. Measurement of Antifolate Drug Levels by Microbiological Assay: Plasma Levels of 2,4-diamino-6-(2-naphthyl)-sulfonylquinazoline, WR-158,122.

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Interim Report No. 14

Measurement of Antifolate Drugs by Microbiological Assay

Mutagenic Studies of Folic Acid Antagonists

Carl C. Smith, Ph.D.
Principal Investigator

Clara S. Genther, M.S.

December 28, 1976

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Compounds which compete with folic acid (folic acid antagonists, FAAs) become limited in their usefulness in the treatment of leukemia, malaria and bacterial infections by the rapid development of resistance. Assays of the plasma levels of certain of these FAAs led to the observation, in about 25% of the determinations, that a higher density of growth of Streptococcus faecium var. durans (ATCC 8043) was obtained at an FAA concentration just below the completely inhibitory level than at one-half this concentration. This and other considerations suggested that FAAs may act not only as selective agents for resistant

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organisms but also as mutagens. Seven FAAs including amethopterin, pyrimethamine, trimethoprim, chlorguanide triazine, an experimental quinazoline, WR-158,122, and two experimental triazines, WR-99,210 and WR-38,839, were tested for mutagenicity in the Salmonella reversion assay developed by Ames. All were found to be negative for strains TA1535, TA1537, TA1538, TA98 and TA100, both with and without microsomal activation. These compounds were then tested as mutagens for three traits in the folic acid-requiring S. faecium. FAAs were shown to cause mutations to folic acid independence, rifampin resistance and FAA resistance. It is postulated that the FAAs induce mutations by causing thymine deprivation in the folic acid-requiring host.

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INTRODUCTION

Compounds which compete with folic acid have been used extensively in the treatment of malaria and leukemia, and in combination with sulfonamides, in the treatment of bacterial infections (5, 14, 29). Resistance to these folic acid antagonists (FAAs) is known to develop readily (18, 23). Although data consistent with a mutagenic role for these compounds have been reported, this development of FAA resistance has generally been ascribed to the selection of spontaneously occurring resistant organisms (25). Hemmerly and Demerec (12) and Iyer and Sybalski (19) have reported low grade mutagenicity of amethopterin and pyrimethamine for the str, pro-1 and trp-6 loci of Escherichia coli. Prophage induction by aminopterin and amethopterin (11), chromosome abnormalities (16, 22, 24) and teratogenic effects (2, 28) have also been described. The planar polycyclic structure of some of the compounds appears similar to that of known DNA intercalating agents, and some of these structures may be subject to epoxide formation. It was decided to investigate further the mutagenic potential of these compounds. The systems used were the Salmonella typhimurium histidine auxotroph reversion assay developed by B. N. Ames (1), and mutation studies of a folic acid-requiring microorganism, Streptococcus faecium var. durans.

MATERIALS AND METHODS

A. Salmonella typhimurium. The mutagen indicator strains were supplied by Dr. B. N. Ames, University of California at Berkeley. They consisted of a missense mutant, TA1535, two frameshift mutants, TA1537 and TA1538, and strains TA98 (TA1538/pKM101) and TA100 (TA1535/pKM101); pKM101 is an R

factor plasmid which makes these host strains particularly susceptible to the effects of certain mutagens (21). All of the strains are deficient in both cell wall synthesis and DNA repair. Stocks were maintained in dimethylsulfoxide at -70°C and were used as described by Ames, et al. (1).

Livers for microsome preparations were obtained from 150 g male Sprague-Dawley rats which had been pretreated by i.p. injection of 50 mg/kg Aroclor 1254 for 5 days and then starved 24 hrs and killed. Homogenate supernatant S-9 fractions were prepared, maintained and applied in activation assays essentially according to the methods described by Ames (1). Livers were homogenized in 0.15 M KCL, 0.05 M Tris HCl, pH 7.4, and the homogenate was then centrifuged 20 min at 9000 g.

B. Streptococcus faecium var. durans. This is Streptococcus faecalis (ATCC 8043) which has been reclassified as S. faecium var. durans (7). It is one of the lactic acid bacteria which requires folic acid and is widely used for folic acid and FAA assay (15, 27). A fastidious organism requiring a relatively complex medium (9), it cannot synthesize folic acid from simple precursors. The requirement for folic acid also can be met by providing pteroic acid, a reduced folate or the products of the folate pathway (20). The organism is a microaerophilic coccus (27) growing in diplo or short chain form in the liquid test medium. Counts of colony forming centers are equated with cell count in this study. The strain was maintained by weekly passage on yeast extract agar.

The medium used was the standard folic acid assay medium as described by Flynn et al., (9) with the exception that glucose and folic acid were sterilized separately and added aseptically at the time of test. This is a semisynthetic medium containing acid-hydrolyzed casein, a vitamin

mixture and purines. For preparation of 10 ml broth cultures, 8 ml quantities of basal medium were sterilized in tubes by autoclaving at 121°C for 10 minutes. Stock solutions of folic acid were sterilized by membrane filtration and were stored in small quantities at -15°C. Dilutions were prepared in sterile 25% glucose solution and were added in 1 ml quantities to yield final concentrations of 2 ng folic acid per ml. This quantity of folic acid allows growth to proceed to approximately 7×10^8 cells/ml in medium without drug. Stock solutions of the FAAs or other test drugs, prepared either in self-sterilizing solution or sterilized by membrane filtration, were diluted in water and added to the tubes in 1 ml quantities to give the final volume.

Solid medium was prepared by adding 2% Bacto agar to the medium described above; as 30 ml gave no advantage, 15 ml in standard 100 mm petri dishes were used routinely. Folic acid, glucose and test drug were added to the sterile agar medium just before pouring.

Growth conditions. The test inoculum for Streptococcus faecium was prepared as follows. The strain was subcultured (1/100) twice in standard medium, and incubated at 37°C in loosely capped 18 mm test tubes without shaking, the first for 24 hours and the second for 18-20 hours. The resulting culture was centrifuged, washed twice and resuspended in sterile saline to form a suspension with turbidity measuring 50 on the Klett-Summerson photoelectric colorimeter (660 nm filter). 0.1 ml samples of this suspension, containing approximately 10^7 cells, either were added to 10 ml culture volumes or were plated. Plating procedures used included standard pour plates, spread plates, or top agar plating in 2 ml of 0.6% molten agar. When spot plates were used, an FAA was added to sterile

filter paper placed on the center of the inoculated plate. Plates were incubated in a loosely covered container to retard evaporation. Growth in liquid cultures was read as turbidity in the colorimeter after 22 hours incubation. Colony counts on plates were made at 24 hours and at intervals thereafter up to 14 days. Colonies were not readily visible until after 48 hours incubation. In experiments where the organism was first exposed to FAA in liquid culture and then plated, a 10^7 cell inoculum was cultured overnight in the presence of the drug. These cells were then washed, a standard inoculum of 10^7 cells was prepared again and was subcultured in standard medium for 18-20 hours to allow expression. Standard inocula were then delivered to selection plates. It was found that approximately ten times as much FAA was needed in agar plates as in liquid medium for the same degree of inhibition. All plate counts reported are averages of at least duplicate plates.

Mutagens and drugs. Folic acid (FA) was obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.; chlorguanide triazine (CGT, cycloguanil, 1-p-chlorophenyl-1, 2-dihydro-2,2-dimethyl-4, 6-diamino-s-triazine) from Parke, Davis and Co., Detroit, Mich.; amethopterin (MTX, methotrexate, 2,4-diamino-N^{10}-methylpteroylglutamic acid) was supplied by Dr. James L. Ruegsegger, Lederle Laboratories; pyrimethamine (PM, 2,4-diamino-5-p-chlorophenyl-6-ethyl-pyrimidine), trimethoprim (TMP, 2,4-diamino-5-(3', 4', 5'-trimethoxybenzyl)-pyrimidine), WR-158,122 (2,4-diamino-6-(2'naphthyl)-sulfonylquinazoline), WR-99,210 (1-(2',4',5'-trichlorophenoxypropoxy)-1, 2-dihydro-2,2-dimethyl-4, 6-diamino-s-triazine), and WR-38,839 (1-(3',4'-dichlorobenzyloxy)-1,2-dihydro-2,2-dimethyl-4, 6-diamino-s-triazine) were supplied by the Division of Medicinal Chemistry,

Walter Reed Army Institute of Research, Washington, D.C. Structures of the FAAs are depicted in Figure 1.

Methyl methanesulphonate (MMS) was obtained from Eastman Chemical Co., Rochester, N.Y.; 2-aminoanthracene (2AA) and N-nitro-N-nitrosoguanidine from Aldrich, Milwaukee, Wis.; and 4-nitroquinoline-1-oxide from Koch-Light Laboratories Ltd., Colnbrook, Bucks, England was provided by Dr. Tong Man Ong of the National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Aroclor 1254 was obtained from Supelco, Inc., Bellefonte, Pa.; bromodeoxyuridine and 2-aminopurine from Sigma Chemical Co., St. Louis, Mo., proflavine from Nutritional Biochemicals, Cleveland, Ohio, and quinacrine mustard (NS3424) from the Cancer Chemotherapy National Service Center.

RESULTS

A. Studies with S. typhimurium. No FAA tested was found to be mutagenic in spot tests using strains TA1535, TA1537, TA1538, TA98 and TA100 without microsomal activation. Since all were somewhat toxic at 100 µg/plate in spot tests, with narrow zones of inhibition indicative of poor diffusion by these compounds, experiments were performed using plate assays incorporating graded doses of the FAA. Data for tests of TA98 and TA100 with and without microsomal activation appear in Table 1. Neither TA98 nor TA100 was reverted to a significant extent by PM, MTX, WR-158,122 or WR-99,210 at the doses indicated. WR-99,210 and WR-158,122 proved to be toxic at higher doses; this toxicity was relieved somewhat by the presence of the microsomal system. All FAAs tested in pour plates were toxic for S. typhimurium tester strains at 100 µg/pl. These same FAAs gave similar

results using strains TA1535, TA1537 and TA1538; that is, no significant mutagenicity occurred in the presence or absence of activation. Furthermore, varying the concentration of S-9 over a range of 25 to 150 µl per plate, or employing uninduced, or phenobarbital, or 3-methylcholanthrene-induced S-9 did not alter the lack of mutagenicity of the FAAs for any Salmonella strain (Schoeny et al., manuscript in preparation).

B. Studies with S. faecium. During the last 3 years we have been using S. faecium var. durans in microbiologic assays of plasma samples for FAA content. In approximately 20-25% of these assays an unpredicted result was obtained. In these cases more growth occurred at higher concentrations of drug than at one-half these concentrations. For example, in 62 out of 338 assays (18% of tests) with WR-158,122 more growth was obtained at 1 ng/ml than at half the concentration, 0.5 ng/ml (Figure 2). With WR-99,210 such results were obtained in 35 of 132 trials (26%). This effect had not been recognized in our earlier studies using PM; however, in reviewing our data a few such results were found with that compound.

Checks of isolates from the tubes having this unexpected growth showed that the organisms had the usual characteristics of Streptococcus faecium var. durans (7) but were approximately 5 times as resistant to FAA as the stock culture.

Mutation to folic acid independence - spot tests. Typically when 10^7 cells of a stock culture of S. faecium are plated in the absence of added folic acid a few folic acid-independent colonies develop. This suggested that perhaps folic acid independence could be mutagenically induced in this strain; so spot tests of various concentrations of FAA or of standard mutagens were conducted on plates lacking folic acid. Rings

of folic acid-independent colonies (see Figure 3) were obtained with bromodeoxyuridine, 2-aminopurine, proflavine, quinacrine mustard, and N-nitro-N-nitrosoguanidine, and with each of the 7 FAAs tested: PM, TMP, CGT, MTX, WR-38,839, WR-99,210 and WR-158,122. Rings were obtained with a wide range of concentrations of the FAAs but not every trial with the known mutagens or with the FAAs yielded positive results (Table 2).

Mutation to FAA resistance - spot tests. Spot tests of the FAAs were also made using plates containing folic acid. Rings of FAA-resistant colonies were formed in the zone of inhibition around the drug spot with the 7 FAAs listed above. Again positive results occurred using a wide range of concentrations of each drug but were not invariably obtained (Table 2). The position of the ring of mutants and the diameter of the area of inhibition varied with the type and amount of FAA used. However, for any given FAA, increasing the amount of compound did not increase the diameter of the ring above a certain level. Apparently, FAAs diffuse too slowly to prevent growth beyond a certain distance.

Mutation to rifampin resistance in broth cultures. Since both growth without folate and resistance to FAA may be related to the folate pathway, an attempt was made to detect unrelated mutagenic effects. Mutation to rifampin resistance was chosen since S. faecium is sensitive to this antibiotic at 2.5 µg/ml on agar plates. Spot tests could not be used in this case, since the combined application of FAA and antibiotic eliminated all growth; resistance to this drug combination would require the formation of a double mutant. For this reason a sequential test was employed in which S. faecium was first grown in the presence of partially inhibitory concentrations of FAA, subcultured in drug-free medium and

then plated on agar for selection for rifampin resistance or for the properties of folic acid independence or FAA resistance. Typical results given in Table 3 show that exposure to FAA increased the number of rifampin-resistant colonies 39 to 115-fold. In other experiments the number of rifampin-resistant colonies has increased up to 4500-fold.

Two different levels of rifampin resistance were observed. Of 710 colonies isolated at 5 μg/ml, 61 percent were able to grow at 100 μg/ml. Others were unable to grow at concentrations above 5 μg/ml.

The possibility was considered that rifampin resistance arose spontaneously in these experiments and that the presence of the FAA in some way favored the multiplication of these rifampin-resistant cells. This was tested in two ways. First, Figure 4 demonstrates that the parent strain, a rifampin-resistant strain, and a strain resistant to both rifampin and chloramphenicol have identical growth rates in the absence of FAA. Secondly, the double mutant strain was mixed with the stock sensitive strain in the proportions of $1/10^5$ and $1/10^4$. These mixtures were cultured in the presence of WR-158,122, expressed in the absence of FAA and plated on rifampin agar. Rifampin-resistant colonies were patch checked for chloramphenicol resistance. It can be seen in Table 4 that the ratio of chloramphenicol-resistant to rifampin-resistant colonies in the mixed cultures decreased with increasing exposure to FAA. This would be expected due to the generation of additional rifampin-resistant cells from among the parent, sensitive population. Thus, it does not appear that the original rifampin-resistant, chloramphenicol-resistant cells had a selective advantage in the FAA medium. It appears, rather, that they may not be able to compete successfully with the wild

type organism in mixed culture. Direct plating for the scoring of chloramphenicol resistance was not possible in this experiment due to an apparent diffusion of the chloramphenicol resistance effect which allowed growth of surrounding cells.

Mutation to folic acid independence and FAA resistance in broth cultures. Cultures exposed to FAA in broth cultures were also checked for numbers of colonies able to grow without folic acid and for the numbers showing FAA resistance. In the results shown in Table 3 the numbers of folic acid-independent colonies per plate ranged from 90-1300 as compared with 19 on the control.

Scoring for FAA resistance is more difficult than scoring for folic acid independence or for rifampin resistance because the FAAs are only bacteriostatic. Comparison of stock and FAA-exposed cultures made at the same time show reproducible increases in numbers of colonies (Table 3) with resistance to FAA.

It would be expected that under our conditions of FAA exposure naturally occurring FAA resistance would be selected. However, attempts to isolate highly FAA-resistant cells from the untreated stock culture were unsuccessful ($<10^{-10}$).

Independence of mutational events. Cross checks by patch test or replica plating to selective media indicate that the three traits, folic acid independence, FAA resistance and rifampin resistance arise independently. As presented in Table 5, 6.5% of the colonies which were folic acid-independent were resistant to FAA, and a separate 6.5% were rifampin-resistant. Of the colonies which were rifampin-resistant, 10% were resistant to FAA and a separate 11% were folic acid-independent. Of

the colonies resistant to FAA none were resistant to rifampin and only 30% were folic acid-independent.

Relation of growth inhibition by FAAs to mutagenesis. The data for the three types of mutants were examined for possible relationships between the amount of growth inhibition of S. faecium and the number of mutants produced. For all three markers it was necessary that growth be inhibited approximately 50% before the number of mutants exceeded 10 times the mutant level of the untreated control.

For folic acid independence, the marker for which there is the most data, there was a direct relationship between the proportionate increase in number of mutants and the inhibition of growth by the FAAs. Data for PM and WR-158,122 are shown in Figure 5. The proportionate increase in number of mutants is plotted versus the amount of growth inhibition produced by the FAAs; dotted lines represent the 90% confidence limits of the response. Available data for FAA and rifampin resistance appear to follow the same pattern.

DISCUSSION

In most cases it is generally recognized that the development of drug-resistant populations in laboratory clones is the result of selection of spontaneously occurring resistant mutants (25). It has been shown occasionally, however, that a growth-inhibitory drug may have mutagenic properties (10). The data presented in this paper indicate that drugs which are folic acid antagonists have mutagenic properties for S. faecium but are negative in tests with S. typhimurium.

Previous studies have indicated low-grade mutagenicity of MTX and PM for E. coli (12, 19) and negative results with MTX on S. typhimurium (13). When the S. typhimurium tester strains of Ames were applied to seven FAAs in our laboratory, the FAAs failed to revert the missense mutant in TA1535 and TA100, as well as the frameshift mutants in TA1537, TA1538 and TA98 when the FAAs were used in spot tests or at any of several concentrations in pour plate assays. That the FAAs were delivered to the bacteria is evidenced by their toxicity for one or more strains at high doses (100 µg/pl). The toxicity was relieved somewhat by the presence of S-9 liver mix. This effect could be due to a sparing action of the folic acid present in the liver fraction. It is also possible that the liver microsomal fraction was able to bind or to metabolize a portion of the drug to some inactive form. In any case, the S-9 mix did not activate the FAAs to a form capable of reverting the Salmonella auxotrophs. This was true both with uninduced or with a wide range of concentrations of phenobarbital-, 3-methylcholanthrene- or PCB (Aroclor 1254)-induced microsomal fractions (Schoeny, et al., manuscript in preparation).

FAAs are far more inhibitory for the folate-requiring S. faecium than for the S. typhimurium (0.01 to 0.10 µg/plate for Streptococcus as opposed to 100 µg/plate for Salmonella). It may be that the failure to mutate the folic acid-independent Salmonella strains is related to their relative insensitivity to these compounds.

Initial evidence for the mutagenicity of FAAs for S. faecium was found in tube assays for FAA bacteriostasis in which more growth occurred at higher concentrations of drug than at one-half those concentrations. When S. faecium was used in spot tests for conversion to folic acid

independence, positive results were obtained with all seven FAAs tested: CGT, MTX, PM, TMP, WR-38,839, WR-158,122 and WR-99,210. Rings of folic acid-independent colonies were obtained at frequencies of 13-54% of trials with various FAAs. Spot tests for FAA resistance were positive at rates of 13-21%. That positive results were not achieved consistently is probably due to the delicate balance of interactions between the metabolism of the organisms as they grew on the plate and the bacteriostatic activity of the FAA diffusing from the test spot. We conclude that for some trials the bacteriostatic FAA disallowed expression of folate independence or FAA resistance. In sequential tests in which S. faecium was first exposed to partially inhibitory concentrations of FAA and then tested for folic acid independence and FAA resistance, the results were more consistent.

Since both markers mentioned above involved the folic acid pathway, it was decided to check the mutagenic effects of FAAs on an unrelated marker. S. faecium is a relatively fastidious organism requiring at least 10 amino acids in addition to other growth factors, so metabolic auxotrophs were not feasible for study. Resistance to rifampin was chosen as a marker since the strain was shown to be quite sensitive to this drug. In assays wherein cells were exposed to FAA, expressed in the absence of FAA, and then plated for selection, the number of rifampin-resistant colonies was increased from 39 to 4500 times over the number in the unexposed spontaneous control. At least two different levels of rifampin resistance were observed in mutants isolated. These results were obtained with all FAAs tested: CGT, MTX, TMP, PM, WR-158,122 and WR-99,210. Sequential exposure was necessary to avoid the additive growth-inhibitory effects of these

compounds. The experiment assumes that previously existing and newly arising rifampin-resistant cells have no selective growth advantage during or after exposure to an FAA. Our data indicate this to be the case for the following reasons.

In the first place, patch-checking and/or replica plating colonies to selective media showed the properties of FAA resistance, rifampin resistance, and folic acid independence to be generally independent events. No colonies selected on FAA plates were rifampin-resistant, and only 10% of colonies growing on rifampin were resistant to FAA. If FAA selection were allowing overgrowth of pre-existing rifampin-resistant organisms, this percentage would be expected to be higher. Interestingly, even folic acid-independent colonies were generally not FAA-resistant, with only 6.5% of such cells having both properties. Levels of folic acid biosynthesis were not examined in these folic acid-independent, FAA-resistant cells. That as many as 30% of the FAA-resistant cells showed folate independence may be due in part to the double exposure these cells received, first in initial exposure for mutagenesis, and second, during isolation as FAA-resistant colonies.

Secondly, as shown by comparative growth curves, there appears to be no selective growth advantage for rifampin resistance or sensitivity in the absence of FAA. The wild type organism, a rifampin-resistant strain, and the strain doubly resistant to rifampin and chloramphenicol all show essentially identical growth rates.

Thirdly, direct seeding experiments were conducted in which rifampin-resistant cells genetically tagged with a chloramphenicol resistance marker were diluted with sensitive cells and carried through the FAA

treatment. This assay showed no indication of selective advantage for rifampin-resistant organisms in the presence of FAA. Thus, the increased incidence of rifampin-resistant cells is not due to selective conditions occurring during the FAA treatment procedure.

It has been established that by interfering in the folate pathway FAAs inhibit the production of thymine, purines, protein and lipid (2). For S. faecium the requirement for folic acid in the growth medium used here can be replaced by the addition of thymine (17). In the suspension assays for FAA mutagenesis a certain level of FAA growth inhibition was shown to be necessary. Although the FAA dose required for bacteriostasis and mutagenesis varied somewhat for individual assays, a dose-response relationship could be shown between growth limitation and numbers of folic acid-independent mutants produced.

We suggest that the FAAs, in competing with the folic acid, produce a thymine insufficiency which, while not completely inhibiting growth, does interfere with DNA metabolism so as to cause mutations. Thymine starvation is known to produce mutations in E. coli (4, 6, 8), B. subtilis (3), and T4 bacteriophage (26). Besides being mutagenic in itself, thymine deprivation could additionally promote mutagenic intercalation of the planar polycyclic FAAs.

That FAAs can effect mutagenesis is of clinical significance. Since the folic acid pathway is common to all cells, the mechanisms operative with S. faecium will probably be similar in other types of organisms sensitive to these compounds. When administered repeatedly at low doses as has been common in the prevention and treatment of malaria, the FAAs are capable of selecting for cells resistant to themselves. On the other hand,

high doses delivered for maximum chemotherapeutic effect may be capable of inducing a variety of mutations.

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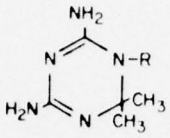
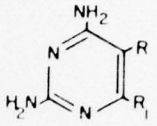
STRUCTURES OF FOLIC ACID ANTAGONISTS



A. s-TRIAZINE



B. PYRIMIDINE

A. s-TRIAZINES

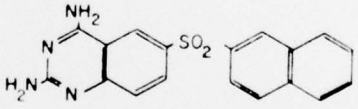
	R	R_1
CGT	$4\text{-}ClC_6H_4\text{-}$	
WR-38,839	$3,4\text{-}Cl_2C_6H_3CH_2O\text{-}$	
WR-99,210	$2,4,5\text{-}Cl_3C_6H_2O(CH_2)_3O\text{-}$	

B. PYRIMIDINES

PM	$4\text{-}ClC_6H_4\text{-}$	$C_2H_5\text{-}$
TMP	$3,4,5\text{-}(CH_3O)_3C_6H_2\text{-}$	H-

C. QUINAZOLINE

WR-158,122



D. PTERIDINE

MTX

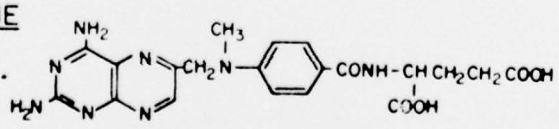


Figure 1

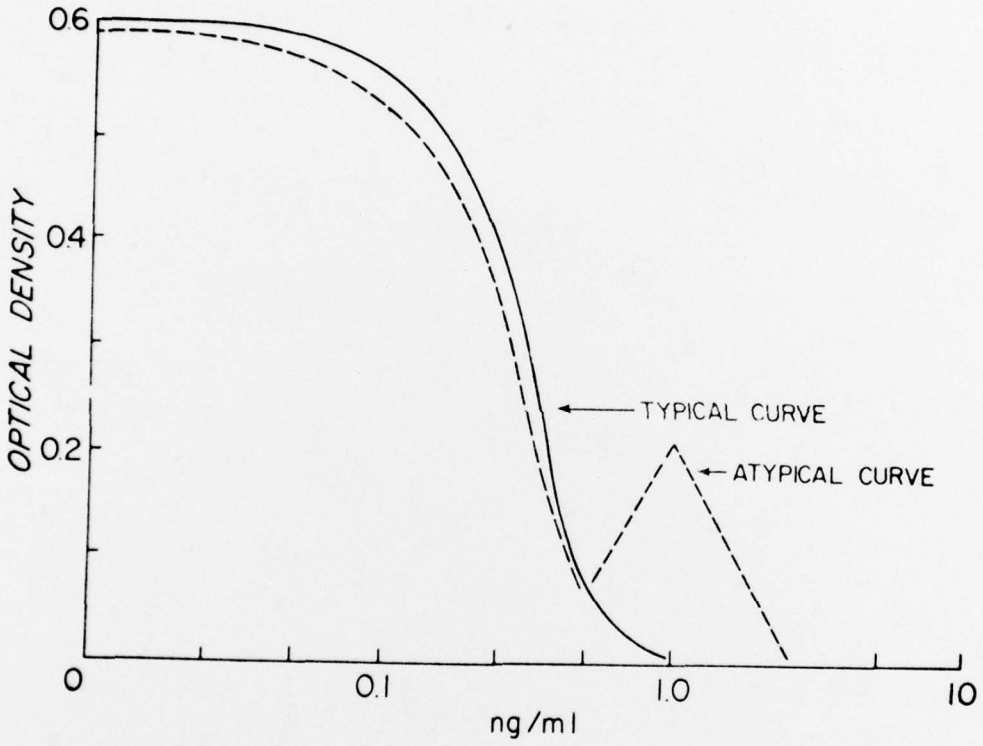


Figure 2. Effects of WR-158,122 on growth of S. faecium.

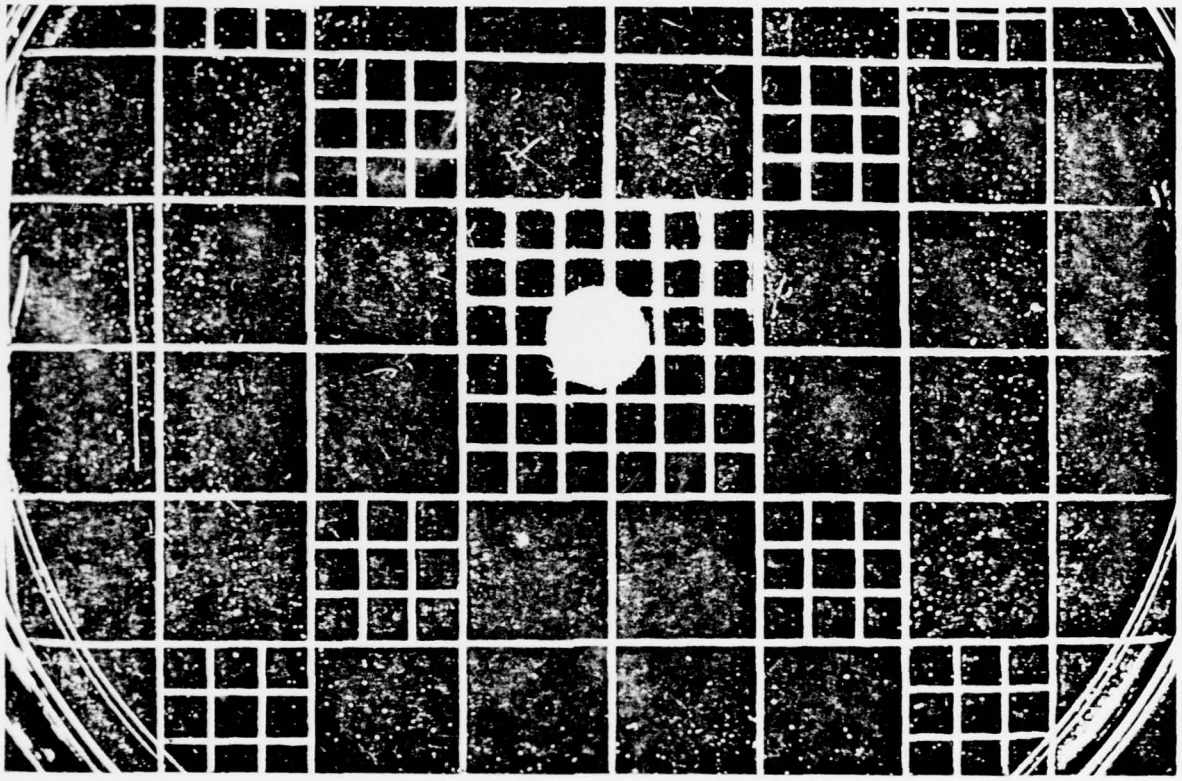


Figure 3. Spot test of 100 μg WR-99,210 with S. faecium on medium without folic acid. Large squares have area of 1 cm^2.

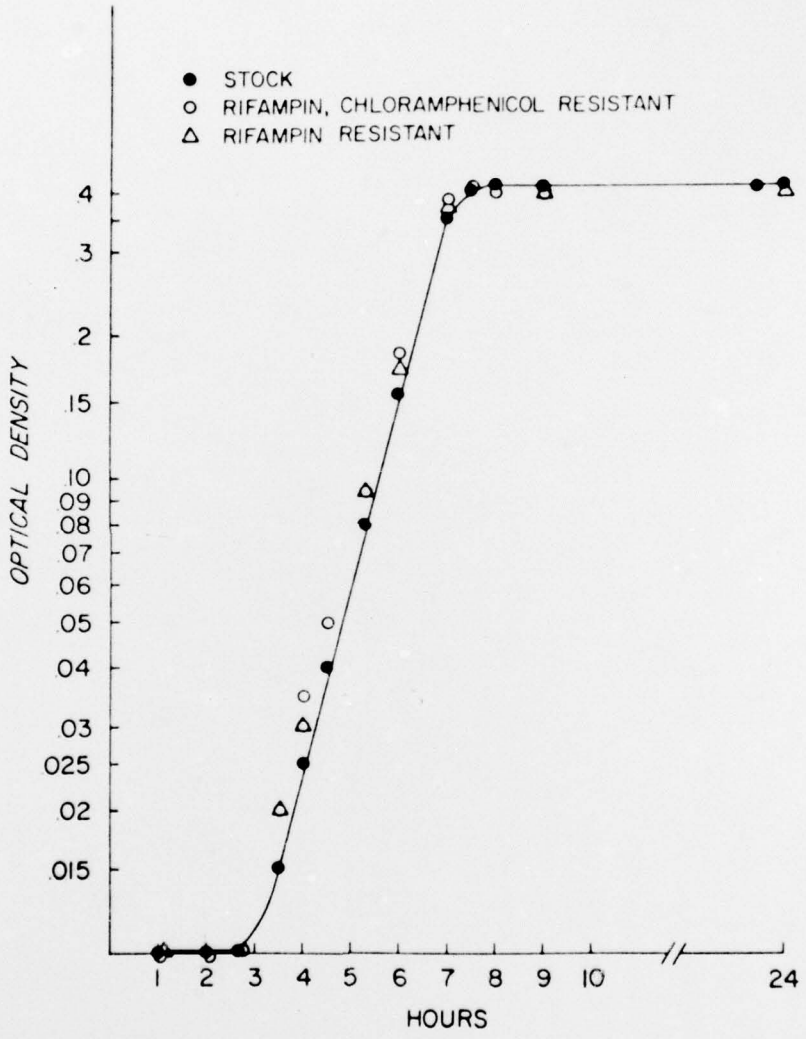


Figure 4. Growth of S. faecium stock, rifampin-resistant and rifampin-resistant, chloramphenicol-resistant strains in test medium.

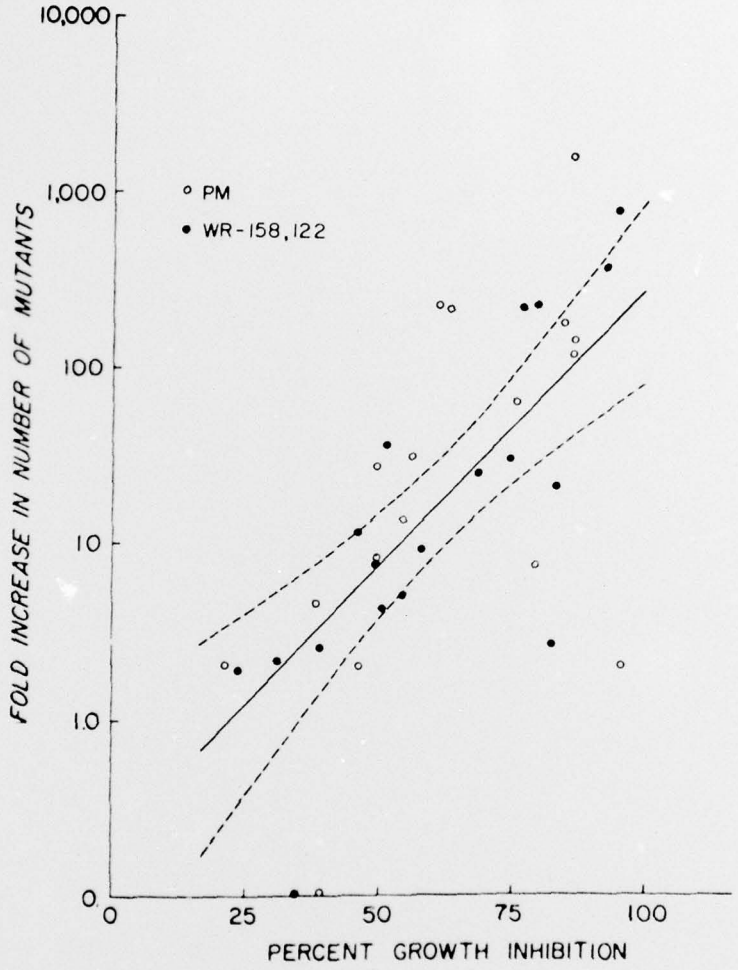


Figure 5. Relationship between fold increase in number of folic acid-independent mutants and degree of growth inhibition of S. faecium by PM or WR-158,122.

TABLE 1. The lack of mutagenicity of folic acid antagonists for S. typhimurium in pour plate assays.

Mutagen	µg/plate	No. of colonies[a]			
		TA98		TA100	
		-S9	+S9[b]	-S9	+S9
None	-	15	27	90	83
2-aminoanthracene	5	30	580	31	814
methyl methanesulfonate	97	ND[c]	ND	1710	ND
4-nitroquinoline-1-oxide	0.2	180	ND	ND	ND
pyrimethamine	100	I[d]	I	I	I
	5	2	0	21	48
	1	3	0	20	55
	0.5	0	3	16	45
	0.1	0	1	37	24
methotrexate	100	I	I	I	I
	10	5	1	15	42
	5	1	3	15	48
	1	7	5	28	36
	0.5	0	6	3	50
	0.1	0	0	24	18
WR-158,122	100	I	I	I	I
	10	I	I	I	I
	5	I	I	I	I
	1	I	I	I	I
	0.5	I	0	I	I
	0.1	3	0	0	23

Table 1 continued

TABLE 1. The lack of mutagenicity of folic acid antagonists for S. typhimurium in pour plate assays.

Mutagen	µg/plate	No. of colonies[a]			
		TA98		TA100	
WR-99,210	100	I	I	I	I
	10	I	I	I	I
	5	I	I	I	I
	1	1	5	10	17
	0.5	5	3	30	55
	0.1	3	3	38	32

[a]Based upon quadruplicate platings. Values for spontaneous are averages, other values are average minus the spontaneous.

[b]S9 was from pooled PCB- induced rat liver homogenate and was used at a concentration of 50 µl/plate.

[c]ND - not done.

[d]I - inhibited growth.

TABLE 2. Results of spot tests with folic acid antagonists on S. faecium

Compound	Folic Acid Independence			FAA Resistance		
	Amount Spotted (µg)	Number of Trials	% Positive[a]	Amount Spotted (µg)	Number of Trials	% Positive
PM	0.3-100	43	40	0.5-50	28	21
WR-158,122	0.5-20	44	55	0.1-10	28	21
WR-99,210	0.3-20	46	54	0.1-10	29	21
CGT	100-500	12	42	100	8	13
MTX	5-1000	15	13	5	3	+[b]
TMP	50-1000	3	+[b]	50-1000	3	+[b]
WR-38,839	1000	1	+[b]	1000	1	+[b]

[a]The positive or negative results were independent of drug concentration used here since both positives and negatives occurred over entire range reported. See text.

[b]All trials were positive.

TABLE 3. Numbers of mutant colonies of S. faecium obtained after exposure to folic acid antagonists in broth cultures

Folic acid antagonist (ng/ml)	Plate Count Selective Agent (ng/ml)						
	Folic acid 2.0	Folic acid 0.0	Rifampin[a] 5,000	WR-158,122[a] 25	WR-99,210[a] 10	PM[a] 100	MTX[a] 10
None	cf[b]	19	0	23	5	0	0
WR-158,122 (0.5)	cf	610	75	1320			
WR-99,210 (0.25)	cf	90	39		1200		
PM (2.5)	cf	1300	55			31	
MTX (0.25)	cf	205	115				hd[c]

[a]Agar also contained folic acid 2 ng/ml

[b]cf - confluent growth

[c]hd - heavy growth but discrete colonies

TABLE 4. Percent of rifampin-resistant, chloramphenicol-resistant colonies when mixtures of sensitive and resistant strains of S. faecium were grown in presence of WR-158,122

Optical density after 22 hours[a]	Number of rifampin^R and % of double resistant/rifampin^R colonies when initial ratio of doubly resistant/sensitive strain was					
	Sensitive Only		10^{-5}		10^{-4}	
	No.	%	No.	%	No.	%
Plated immediately	1	(10)	200	(100)	4000	(100)
0.5	1	(0)	140	(76)	344	(60)
0.4[b]	8	(10)	100	(78)	555	(50)
0.3	8	(10)	233	(50)	686	(40)
0.1	4	(25)	609	(44)	4675	(4)

[a] When the double resistant strain was exposed alone to these conditions, all the colonies remained doubly resistant.

[b] Increasing concentrations of WR-158,122 were employed so as to retard growth.

TABLE 5. Independence of Mutational Events

Phenotype Selected	Number Tested	Percent of colonies showing Folic Acid Independence	Folic Acid Antagonist Resistance	Rifampin Resistance
Folic acid Independent	168	100	6.5	6.5
Folic acid Antagonist Resistant	257	30	100	0
Rifampin Resistant	99	11	10	100

Signature Page

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Signature

December 28, 1976
Date

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